EXPRESSION AND ROLE OF TISSUE TRANSGLUTAMINASE IN LEUKOCYTES IN MULTIPLE SCLEROSIS AND EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Chapter 6

SUMMARIZING DISCUSSION

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MS is a seriously incapacitating progressive neuroinflammatory disorder for which potent and tailored pharmacological treatments are needed. One of these treatments is Natalizumab, which is very effective in blocking T-lymphocyte infiltration into the CNS and reducing clinical symptoms. Unfortunately, it can also cause serious side effects [307, 308]. This example of a very effective treatment demonstrates that drugs that reduce the influx of leukocytes or specific subsets thereof, but with fewer side effects, are of significant interest in MS research. With this goal in mind, a detailed understanding of the type of cells and mechanisms involved in the disease process of MS is of crucial importance to develop new therapeutic agents to halt or dampen disease progression. Based on the outcome of previous research, we believe that especially monocytes and/or macrophages are an interesting target. They are known to have a detrimental influence in the early stage of MS pathogenesis and in matching inflammatory animal MS models (experimental autoimmune encephalomyelitis, EAE) and constitute the majority of infiltrated immune cells in the CNS lesions [212, 225, 226, 254, 309]. Thus, monocytes and macrophages are of special interest as loci of targets for the development of tailored treatments for MS.

Within this context, we focused on the enzyme TG2 and its potential role in MS and EAE. The increased presence and activity of TG2 during inflammation *in vivo* is known to contribute to many fundamental and pathology related processes, including cell adhesion and migration, ECM deposition, cytoskeletal rearrangement, cell differentiation, apoptosis and phagocytic processes such as efferocytosis. The involvement of TG2 in at least some of these processes, also relevant in MS, makes it an interesting protein to study.

The aims of this thesis were therefore to

1) identify the presence of TG2 and the cell types expressing it in post-mortem CNS material of MS patients and of relevant MS animal models, i.e. EAE in rats and mice.

2) determine the contribution of TG2 to EAE symptoms and pathology and its potential as a druggable target.

TG2 expression in MS and EAE lesions

Our studies demonstrated the cellular expression pattern of TG2 in inflammatory active white matter of both post-mortem MS and EAE CNS tissue (**chapters 2, 3, 4 and 5)**. We first confirmed TG2's constitutive expression in endothelial cells in the human and murine CNS tissue, irrespective of the disease state. In post-mortem MS patient-derived brain material, we observed additional cellular TG2 expression, distinct from that of healthy control subjects (**chapters 2 and 3**). Cells with a round morphology present in the perivascular cuff and the surrounding parenchyma of (chronic) active MS white matter

lesions express TG2. These cells are not observed in healthy control tissue or in inactive MS lesions in which inflammatory activity is not detectable anymore. The appearance of TG2 in cells with a round morphology in lesions with inflammatory activity is in line with observations that TG2 expression can be regulated by various inflammatory mediators [310, 311]. The presence of these inflammatory mediators in the CNS is highest during active disease and recedes over time when blood brain-barrier functions are restored and thus less immune cells and inflammatory mediators appear in the brain [238]. We identified these TG2 expressing cells in MS lesions as MHC-II positive cells (**chapter 2**) and further characterized them as leukocytes (i.e. CD45 expressing cells, **chapter 3**). Consequently, we believe that these cells are predominantly immune cells extravasated from the bloodstream [312]. Our more detailed analysis exposed their identity primarily as monocytes and macrophages (**chapter 3**). Although a large portion of cellular infiltrates in MS lesions consists of lymphocytes [216], no TG2 immunoreactivity was found in lymphocytes, indicating that involvement of TG2 in MS is probably exclusively mediated by monocyte- and macrophage-derived TG2.

To further extend our knowledge on TG2 expression in MS pathology, we studied tissue from EAE animal models, which are models commonly used for MS research [313]. Corresponding to the expression of TG2 in macrophages in (chronic) active MS lesions, TG2 appears in macrophages and/or microglial cell in the spinal cord of dark agouti rats suffering from chronic relapsing EAE (cr-EAE, **chapter 2**) and in C57BL/6 mice suffering from monophasic EAE (**chapters 4 and 5**). Interestingly, in both rodent species, the number of TG2 expressing cells in the lesions seems to correlate with lesion size and disability symptoms, supporting a connection between TG2 and inflammation. In addition, we not only observed TG2 expression in affected spinal cord tissue but also in round cells adhered to the luminal wall of the spinal cord vasculature potentially on their way to infiltrate the CNS tissue (**chapter 5**). This indicates that TG2 upregulation in monocytes in EAE might already occur previous to their extravasation into the CNS parenchyma. Circulating monocytes have a relatively low TG2 expression, but upon adhesion to the endothelium, TG2 expression in monocytes immediately increases as they differentiate into macrophages [244]. However, we cannot exclude that TG2 expression is already increased in circulating monocytes in EAE affected animals. An option that is supported by MS patient-derived data, which showed that TG2 expression is higher in circulating monocytes of MS patients compared to those of healthy control subjects [240].

The MS and EAE-related TG2 upregulation in peripheral monocytes might then very well be a prelude to further involvement of TG2 in MS and EAE pathology in the CNS. Indeed, we found that TG2 in CNS lesions in MS and EAE seems to be predominantly expressed in macrophages with lysosomal and therefore phagocytic activity (CD68 expressing macrophages), suggesting that TG2 might be involved in phagocytosis of myelin debris or apoptotic cells [246], an important process for clearance of MS lesions. Unfortunately, further analysis into macrophage phenotypes subsets did not reveal a predilection for any cellular subset in MS lesions (**chapter 3**). Generally, lysosomal active macrophages have an anti-inflammatory signature [248], and there is an increase of TG2 expression in such cells *in vitro* [237, 251]. However, we could not confirm that TG2 expression is limited to an anti-inflammatory macrophage phenotype *in vivo*. These

observations are supported by previous data on the presence of intermediate phenotypes in MS lesions as a substitute for clear distinct cellular macrophage subtypes [38].

We demonstrated that TG2 in rodent EAE and human MS lesions is predominantly expressed in leukocytes and more specifically in macrophages, which are abundantly present in MS lesions and are involved in disease pathology following infiltration of the CNS [179]. Additionally, in another EAE model in marmoset (i.e. non-human primates), showed a similar TG2 expression pattern in monocytes/macrophages [231]. The constraint of TG2 expression to cells associated with lesions that show inflammatory activity in MS and EAE is in line with its upregulation during inflammation. It also supports our hypothesis that TG2 is involved in both disease pathologies, possibly via a contribution to cellular migration and CNS infiltration, a well-known role of TG2 [119], and is involved in the myelin phagocytosis process. The restriction of TG2 expression to specific cell types that are major players in disease pathology of MS and EAE, makes TG2 an interesting therapeutic target with a restricted range of action and potentially limited side effects.

A contribution of TG2 to EAE development in rodents

To investigate a potential causal contribution of TG2 to EAE motor symptom development and pathology, we pharmacologically manipulated TG2 activity in rodent EAE models (**chapters 2 and 5**). For this purpose we used three small molecule compounds that have been developed as irreversible TG2 inhibitors, i.e. KCC009 [181], ERW1041E [153] and BJJF078. In addition, we included two reversible acting competitive substrates, cystamine [200] and monodansylcadaverine [181]. We treated rat and/or mice suffering from experimentally induced EAE with these compounds and assessed the consequence(s) of these interventions on motor symptoms and spinal cord pathology which we supported with additional data derived from an *in vitro* blood-brain-barrier model (**chapter 2**). Surprisingly, the effect of treatment is vastly dependent on the combination of compound and EAE model, which we believe depends not only on the compound used but also on the variation in pathology in different EAE models and/or species. Overall, the pharmacological blockade of TG2 activity in rat and mouse models showed that it can drastically reduce EAE motor symptoms and pathology. The administration of TG2 inhibiting compounds, i.e. KCC009, ERW1041E, cystamine and monodansylcadaverine, resulted in a reduction of motor symptoms, confirming a role for TG2 in EAE. These data on the contribution of TG2 to EAE motor symptom development were supported with data derived from TG2 knockout mice, which similarly to the drug-treated animals, developed less paralysis compared to wildtype littermates (**chapter 2**). Our findings therefore suggest that TG2 is indeed contributing to the development of EAE symptoms in our rodent models. In contrast, the compound BJJF078, did not result in any measurable effects, which we believe might be due to limited bioavailability under our experimental conditions, which has not been tested yet.

The effect of reversible and irreversible compounds was comparable, albeit, on average, irreversible inhibitor treatment seemed slightly more effective. This could be due to their general pharmacological characteristics. Irreversible inhibitors bind covalently to

their target, whereas reversible inhibitors bind non-covalently and therefore may dissociate off the active enzyme and therefore result in less inhibitory action.

In some of our animal experiments, the reduction in motor symptoms was accompanied by a massive reduction in CNS infiltration of leukocytes and lesion formation (**chapter 2**). The animals treated with TG2 inhibitors showed considerably fewer and smaller spinal cord lesions compared to untreated animals suffering from EAE. The cells whose CNS infiltration was hampered by TG2 inhibition were exclusively MHC-II positive cells, presumably monocytes/macrophages. This effect was strongest in KCC009 treated cr-EAE rats and was manifested as a selective reduction in macrophages found in spinal cord lesions. The reduction of specifically this cell type is in agreement with our previous data on their TG2 expression status in EAE. We did not observe any change in lymphocyte infiltration, as we already expected from their lack in TG2 expression (**chapter 2, 3**). Although EAE and MS are considered T cell-driven diseases [203] and TG2 is known to be involved in T cell-mediated cytokine expression [198, 314], our data propose that the effect of TG2 inhibition on EAE motor symptoms is due to monocyte- and macrophage-derived TG2. The effective inhibition of macrophage CNS infiltration in spinal cord lesions after KCC009 treatment may, in turn, explain the observed decrease in demyelination and reduced disability scores of the animals (**chapter 2**). Similarly, complete depletion of macrophages in another rat EAE model did not affect CNS T cell recruitment [225]. However, demyelination was highly reduced [225], which indicates that monocytes/macrophages are indeed important effector cells involved in demyelination in EAE. Our combined data on TG2 expression in monocytes and macrophages (**chapter 2, 3, 4 and 5**) and from the treatment of cr-EAE rats with KCC009 (**chapter 2**) hint towards monocyte- and macrophage-derived TG2 as an important factor involved in their infiltration into the CNS during EAE/MS. However, ERW1041E, a compound that did not clearly inhibit cellular CNS infiltration, still resulted in a reduction of motor impairment in mice (**chapter 5**), but is more modest than found in the cr-EAE model in rats. The mechanism of action behind the reduced symptoms in the mouse model has not been unraveled yet. We consider that the lack of effect of TG2 inhibition on cellular infiltration might be due to a reduced macrophage dependence of the mouse models compared to rat cr-EAE. Comparing the pathology of our EAE models reveals that the cr-EAE model is mainly macrophage driven [67], whereas the mouse models are more T cell driven [68]. Our observations of pharmacological TG2 inhibition in rats versus mice are in line with these described differences in pathology. Nevertheless, we still observe reduced motor symptomatology in mice lacking TG2 or after pharmacological inhibition of TG2 activity. We propose this might be due to a similar effect as the clinic-radiological paradox in MS patients: formation of new lesions and disability scores do not necessarily correlate [315], a paradox that has also been discussed in an EAE animal model [316]. Our observation on the clinical versus lack of histological effect of TG2 inhibition might therefore not only rely on the inhibition of monocyte or macrophage infiltration. One such potential effect could be that TG2 inhibition results in a different composition of cellular subsets of leukocytes or other cell types present in the lesions or a changed activation states and subsequently changed functions of the cells in the lesions. This might directly affect the development of pathology and result in reduced motor symptoms. Other possibilities are not influenced by

reduced cellular migration but could rely on changed signaling pathways by TG2 inhibition, influencing symptom development such as secretion of inflammatory modulators.

Our data suggest that TG2 can contribute to clinical EAE development via not yet unraveled mechanisms. Promising is that our effective therapeutic treatment approach after the onset of disease symptoms resembles the treatment of RRMS patients. Therefore we conclude that the administration of TG2 inhibitors can reduce ongoing inflammation and motor impairment in EAE and hence TG2 could be a potential target of interest in MS patients.

(sub)cellular localization and function of TG2 in EAE

When focusing on TG2 as a target it is essential to consider that it is a multi-functional protein present in several (sub)cellular compartments and that it shows localization-dependent activities [85, 93, 317]. TG2 is constitutively expressed in many cells, including monocytes/macrophages and CNS resident cells. Various stimuli such as lipopolysaccharide and several cytokines including IFNγ and IL-4 [310, 311, 318, 319] lead to a quick upregulation of TG2 expression in monocytes and macrophages which can usually be detected at both the mRNA- and protein level and by measuring enzymatic transamidation (a.k.a. cross-linking) activity which is often observed to be increased under inflammatory conditions [108]. As such, the restriction of TG2 expression to active and chronic active MS and EAE lesions (**chapters 2, 3, 5**) underlines the inflammation related expression of TG2 in active MS and EAE. In general, the predominant (sub)cellular localization of TG2 is in the cytoplasm but its presence has also been confirmed in virtually every other cellular compartment, including the nucleus, mitochondria, endoplasmic reticulum and cell membrane. In addition, an excreted form of TG2 can be found in the ECM [101-104, 106, 107, 320].

Although the reduction in TG2 activity demonstrated clear beneficial effects in our animal models, it remained uncertain which cellular localization of TG2 instigated the observed *in vivo* effects. There are also several possibilities on how this effect was mediated. One of them is that this was due to the actual inhibition of the transamidation activity of TG2 (and/or other TGs) or that it was the result of the down-tuning of other, non-enzymatic functions of TG2. Furthermore, the inhibition might affect the conformation of TG2 and keep it in its open, enzymatically active conformation and therefore affect its (trans-)location and interaction with potential binding partners. We addressed some of these questions by studying several of the above described TG2 inhibitors *in vivo* and *in vitro*. With an *in vitro* blood-brain-barrier model, we confirmed that rat monocyte/macrophage adhesion and migration across rat brain endothelial cells is TG2-dependent (**chapter 2**). In this set-up, we stimulated monocytes with pro-inflammatory cytokines, which are also present in MS and EAE, and observed increased TG2 expression on the cell surface, which then, in turn, could be pharmacologically inhibited by KCC009, resulting in reduced transendothelial migration, a process that might be similar *in vivo*. We subsequently analyzed the specificity of other TG2 inhibitors and observed that they all preferentially inhibited TG2 activity over the other members of the transglutaminase enzyme family (**chapter 5**). Our *in vitro* assays moreover revealed that

the inhibitory compounds vary in their cell permeability, with KCC009 [321] and ERW1041E [181] being the most cell-impermeable TG2 inhibitors. Using the cell-permeable and impermeable TG2 activity inhibitors in our *in vivo* EAE experiments (**chapters 2 and 5**), the cell-impermeable TG2 inhibitory compounds (KCC009 and ERW1041E) were more effective than a cell-permeable one (BJFF078) in reducing EAE motor symptom development and in cell migration *in vitro* (only KCC009), which suggests a potentially important role of extracellular TG2 activity in these processes.

Even though it seems that extracellular TG2 activity is of importance for the here studied processes of cellular migration and development of EAE symptoms, we cannot rule out that intracellular TG2 is involved in the observed effects of TG2 inhibition. Likewise, TG2 is known to also mediate so-called outside-in signaling which means that extracellular TG2 can affect intracellular processes [122, 322], which can be affected by TG2 inhibition. We showed, that KCC009 treatment of *in vitro* macrophages reduced the F-actin stained cellular protrusions, an intracellular process necessary for the regulation of cytoskeletal flexibility. This flexibility is essential for adhesion, migration and extravasation of monocytes and macrophages [206]. The small GTPase RhoA is a major player in this process [134], whose activation is mediated by TG2 transamidation **(chapter 2)**. RhoA activation is only one example that shows how the enzymatic transamidation activity of TG2 can affect cellular motility and migration. How this intracellular process is blocked when KCC009 cannot permeate the cell remains unclear at this point but is presumably due to the above mentioned outside-in signaling. A potential mode of action of extracellular TG2 to affect the cellular protrusions in a similar way as RhoA is focal adhesion kinase (FAK). FAK is a kinase that is activated by the clustering of integrins on the cell surface. This clustering is facilitated by cell-surface TG2 that engages with integrins and direct association of FAK activity regulated by TG2 was shown in pancreatic cancer cells [323]. Accordingly, increased expression of TG2 results in increased integrin clustering and hence FAK activation which itself is associated with a pro-migratory profile of (cancer) cells [324, 325]. Although our study focused mostly on inhibiting TG2 transamidation activity, this process described above is independent of the cross-linking activity of TG2 [325].

Next to its enzymatic activities, some of TG2's actions are non-enzymatically mediated. These enzymatic activity-independent mechanisms can thus be potentially affected by TG2 inhibitory compounds used in this thesis. Of particular interest is the interaction of TG2's fibronectin-binding domain with extracellular fibronectin. The interaction of cells with this extracellular matrix protein is also of relevance for the adhesion and migration of cells [182]. The likely extracellularly mediated effect on macrophage adhesion and migration by KCC009 may thus be, at least partly, due to inhibition of the interaction of TG2 with fibronectin. Although not tested for this compound, the chemically similar compound ERW1041E did not affect TG2-fibronectin binding *in vitro* (**chapter 5**) but still reduced EAE motor impairment *in vivo*. These data suggest that an interplay of TG2's transamidation function and possibly other functions of TG2, that have not been studied in this context, are likely involved in EAE pathology and symptom development.

Visualizing monocyte behavior in EAE – Association of migration with TG2?

Our *in vivo* data of cr-EAE rats treated with the TG2 inhibitor KCC009 showed a clear reduction in the influx of monocytes into the CNS. Moreover, experiments using an *in vitro* blood-brain barrier revealed that TG2 expressed by monocytes is indeed of relevance for these cells to adhere and migrate over the blood vessel endothelium (**chapter 2**). This suggests that TG2 expressed by monocytes plays a role in their *in vivo* crawling behavior along the blood vessels in the CNS as an initial step to migration. As a first proof of principle we created an experimental setup of intravital microscopy to visualize *in vivo* crawling behavior of monocytes and their interaction with the spinal cord endothelium in EAE mice (**chapter 4**). Mounting a permanent spinal cord window [266, 267] allows studying monocyte adhesion and their migration during EAE using intravital two-photon microscopy (IVM). We visualized monocyte behavior in the spinal cord vasculature during EAE in a time-dependent manner by using CX3CR1 transgenic mice, which contain monocytes, macrophages and microglial cells that express green fluorescent protein (GFP). Very few crawling cells were observed in a naive animal and the number and intraluminal crawling duration of the green fluorescent monocytes was highly increased after immunization. The number of these cells remained elevated and showed extended migratory track length during ongoing clinical EAE disease when compared to adjuvant only injected mice or asymptomatic animals. Therefore it seems that during EAE an extended and potentially firmer interaction of these cells with the endothelium occurs, which might be mediated by soluble or cell surface mediators such as chemokines and cytokines [280, 282, 283], but also TG2 could be involved in this process as suggested by our *in vitro* data (**chapter 2**). Post-mortem analysis confirmed TG2 expression in green labeled microglial cells, monocytes and macrophages in spinal cord EAE lesions and even in cells attached to the luminal side of the vasculature. This indicates that TG2 expression already occurs before extravasation into the parenchyma. TG2 might thereby contribute to adhesion and crawling of monocytes, facilitating extravasation into the CNS in this model. Moreover, this data, together with the early observation of extended monocyte crawling on the spinal cord endothelium (**chapter 4**) and early CNS infiltration [274], leads to the conclusion that interference with monocyte adhesion, by e.g. inhibition of TG2, should be applied at a very early stage of EAE and possibly MS, to effectively combat subsequent pathology. However, therapeutic treatment of EAE animals with TG2 inhibitors after the onset of motor symptoms (**chapters 2 and 5**), still reduced symptom development and suggests that even administration of such treatment during ongoing inflammation, might have beneficial effects. A final proof of interest would be to induce EAE in the transgenic mice, treat them with a TG2 inhibitor and visualize the crawling behavior of monocytes during disease to see if indeed at early stages of EAE, TG2 is involved in the interaction of monocytes with the brain endothelium. If so, this might support our hypothesis of TG2 being a potential target to reduce CNS infiltration in MS. Another very interesting approach of visualization could be the use of fluorescently labeled inhibitors that can give direct information on the time and location of active TG2 in this model, which will provide unique and valuable insight into the mode of action of TG2 in EAE.

With the here used intravital microscopy setup in EAE animals, we laid a ground stone to image monocyte adhesion and migration in the spinal cord *in vivo*. This can be used to combine studies on the effect of TG2 inhibitory compounds (and others) in EAE and the cell types involved.

TG2 in cells in MS and EAE lesions: macrophages only?

In a healthy brain, an intact blood-brain barrier excludes the majority of blood immune cells from the CNS. During MS, however, the integrity of this barrier fails and CNS infiltration of leukocytes from the bloodstream occurs, which can have detrimental effects in the CNS [326]. Therefore, inhibiting the cellular influx into the CNS is of interest for MS treatment (and possibly other neuroinflammatory diseases) to reduce the concomitant tissue damage associated with the inflammation and thereby reduce disease pathology and symptoms. Especially the inhibition of macrophage infiltration is one aspect of treatment that could supplement the currently available MS treatment, which mainly focusses on the blockade of lymphocyte infiltration into the CNS.

Inflammatory processes in the CNS during MS do not only attract cells from the bloodstream but also recruit residential cells such as glial cells [160, 327]. It is assumed that these cells exert various roles in MS pathology, including reduction and containment of inflammation, cleaning up debris and the repair of CNS damage. Therefore, when targeting TG2 as a potential factor to limit monocytes and macrophage infiltration in MS, the effects on pathology involving resident CNS cells need to be taken into account. TG2 is known to be expressed in various glial cell types either constitutively or upon stimulation, e.g. microglia [318], astrocytes [180, 328, 329], oligodendrocyte precursor cells [330] and neurons [76]. All these cell types are involved in MS pathology and found in MS lesions [224]. Thus, whereas the data presented in this thesis indicate that TG2 inhibition might improve EAE symptoms and reduce monocytes and macrophage infiltration of the CNS, the effect on other cell types could have other outcomes. Resident microglia cells act similar to monocyte-derived macrophages in EAE and MS and therefore TG2 inhibition should reduce their migration towards the lesion site, which would help dampen the inflammation in early disease but reduce the phagocytic clean-up capacities of microglia. Also, remyelination can be indirectly affected by microglia-derived TG2 as it stimulates oligodendrocyte precursor cell maturation. This results in functional remyelinating oligodendrocytes, which help to revert the damage of demyelination and this process was hampered by TG2 inhibition [330, 331]. However, despite TG2's role in oligodendrocyte maturation, recent data from our group showed that this is a process limited to the developing human brain and is absent in remyelinating MS lesions [332]. Nevertheless, in a demyelinating mouse model, Tgm2 knockout resulted in impaired remyelination compared to TG2-expressing animals [330] and serves to demonstrate that more research is needed in this area.

Another highly important process in MS pathology is gliosis, the formation of the so-called glial scar, a fibrosis like process. Astrocytes are the main contributors to the glial scar which function is to reduce cellular accessibility of demyelinated areas for the purpose of reparation and therefore hampers remyelination and axonal re-growth [329,

333]. TG2's wide variety of substrates include numerous extracellular proteins, including fibronectin [334, 335], laminin [106], vitronectin and fibrinogen [336] that are involved in gliosis and fibrosis and especially TG2's role in fibrosis has been studied extensively [106, 137, 337]. Crosslinking of the above-mentioned proteins in gliosis and fibrosis increases their proteolytic, chemical and mechanical resistance and also their stiffness. The latter one is thought to assist cell adhesion and migration but also helps closing off lesion areas in gliosis [119, 127]. Therefore, inhibiting TG2 might lower the rigidity of the extracellular matrix and thereby reduce glial scar formation which in turn may increase tissue reparation due to better accessibility of lesions by cells involved in remyelination.

Concluding remarks and future outlook

The data presented in this thesis show that TG2 is expressed in monocytes and in macrophages in MS and EAE lesions and can contribute to the development of symptoms and cellular CNS infiltrates in EAE affected rodents. Pharmacological TG2 inhibition revealed the contribution of TG2 activity to EAE. Its inhibition cannot only reduce macrophage infiltration into the CNS but also reduce lesion formation and paralysis development in EAE affected rodents. We propose that TG2 inhibitory treatment as early as possible will result in the best treatment outcomes in EAE and potentially MS by preventing cellular CNS influx and associated CNS damage. Although our data are promising and EAE is a broadly used animal model for MS research and resulted in currently available valuable MS treatments, it is possible that the situation of TG2 contribution to pathogenesis in MS is different. For that reason, prior to drawing firm conclusions on TG2 as a druggable target in MS, further research is needed. Using primary cells derived from MS patients as well as analyzing CNS biopsies of new and active lesions instead of post-mortem tissue can confirm if our animal-derived data can be transferred to MS patients.

We provide exciting initial data on the contribution of TG2 to EAE development but further research is needed to unravel the contribution or various TG2 expressing cell types and how their functions are affected by TG2 inhibition. Especially the time-dependent factor of inhibiting TG2 during disease progress might result in diverse outcomes of TG2 inhibition. Our data indicated that inhibition of TG2 in early disease phases before massive cellular CNS infiltration has occurred, might be beneficial to reduce this infiltration. However, at later disease stages when cellular infiltrates are already present in the CNS, it might hamper necessary repair mechanisms and cleanup of debris in the lesions. Special attention should also be paid to the cellular locus of action of TG2 inhibition. Our encouraging data with cell-impermeable TG2 inhibitory agents suggests that extracellular TG2 facilitates the migration of macrophages and therefore their CNS infiltration. Furthermore, the development of new and highly specific inhibitors that only target single mechanisms of action of TG2 will greatly contribute to our knowledge of the specific functions of TG2 in EAE and, more importantly, enable proper evaluation of the enzyme's potential as a future therapeutic target in MS. TG2 enzymatic transamidation activity is up to now the focus of these efforts, but specific inhibitors focusing on the interaction of TG2 with extracellular matrix proteins such as fibronectin to study the effect

of TG2 on monocytic transendothelial migration have a large potential and would aid the evaluation of TG2 and its contribution to EAE and MS. Moreover, this is not only of interest for MS patients but also for other diseases that are associated with TG2-ECM interactions and cellular migration like, for example, atherosclerosis and cancer metastasis [338, 339].